Correlation of Ultrastructure in *Azotobacter vinelandii* with Nitrogen Source for Growth

JOEL OPPENHEIM AND LEON MARCUS

Department of Microbiology, Loyola University (Chicago), Stritch School of Medicine, Maywood, Illinois 60153

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*Azotobacter* synthesizes an extensive internal membranous network when grown with air (N₂), i.e., under conditions when these bacteria fix nitrogen. Very slight quantities of internal membrane, concentrated mainly about the cell periphery, are formed when *Azotobacter* grows with fixed nitrogen, i.e., ammonia and amino acids. Compared to cells growing with ammonia, cells utilizing atmospheric nitrogen as the sole nitrogen source are smaller in size and volume, grow one-third slower, and lack detectable poly-β-hydroxybutyrate.

Microbial biomembranes not only comprise a vital part of the structure of the cell envelope but also have been implicated in several vital functions of the cell, e.g., respiration (5), function of the nucleus (6, 12, 22), cell division and cell wall synthesis (7, 11, 23), permeability (15, 16), ribosomal attachment (1, 2, 8, 24, 25), photosynthesis (3, 9, 10, 26), and nitrogen fixation (4).

Invagination of the cytoplasmic membrane of *Azotobacter* has been described by Wyss et al. (27). These observations were extended with the publication of the electron micrographs of Pangborn et al. (19), in which an extensive inner network of membranes is depicted. This report details the effect of the nitrogen source on the induction of the internal membranous network.

**MATERIALS AND METHODS**

One-liter cultures of *A. vinelandii* strain OP were grown in a modified Burk's nitrogen-free medium containing, per liter: KH₂PO₄, 0.2 g; K₂HPO₄, 0.8 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.4 g; Fe₂(SO₄)₃, 1 mg; NaMoO₄, 0.1 mg; and sucrose, 20 g. The cultures were shaken on a New Brunswick rotary shaker at 32°C in 2-liter flasks fitted with stainless-steel baffles to increase aeration (18). Fixed nitrogen was added as either 0.5% Casamino Acids (enzymatic digest), 0.25% NaNO₃, or 0.25% NH₄Cl to the basic Burk's medium. Growth rates were determined by removing samples at hourly intervals after the initial inoculation and reading appropriate dilutions in a Beckman DB spectrophotometer at 600 nm.

All cultures were harvested during exponential growth. Immediately before harvesting, 40-ml samples of each culture were removed from the culture and prepared for electron microscopic analysis. The remaining portions of the cultures were harvested by pouring the flask contents onto finely crushed ice. The cells were sedimented in a refrigerated centrifuge and suspended in cold AVO buffer [tris(hydroxymethyl)aminomethane-hydrochloride, 0.05 M (pH 7.2); magnesium acetate, 0.004 M; KCl, 0.1 M; and spermidine·3HCl, 0.002 M] containing 3.0 g/m glucose. The cells were then centrifuged and suspended in a minimal quantity of the same buffer. Lysis of the cells was accomplished by abruptly adding a 10-fold quantity of AVO buffer to the glycerol-laden cells by the method of Robrish and Marr (21). A 100-μg amount of electrophoretically pure deoxyribonuclease was included in the buffer used for osmotic lysis.

A modified sucrose density gradient procedure was used to separate intact cells from lysed cells. After breakage, the suspension was held at 0°C for 15 min. The soluble cellular contents were separated from intact and broken cells by low-speed centrifugation. After the supernatant fluid was decanted, the pellet was washed twice by centrifugation. The pellet was suspended in a minimal volume of buffer, placed on a 20 to 70% sucrose density gradient, and centrifuged for 10 min at 10,000 × g in an SB 110 swinging bucket rotor in an IEC B-20 centrifuge. During centrifugation, two discrete bands were formed within the gradient. The band closest to the top of the tube, by electron microscopy, was shown to contain lysed cells exclusively. The band closest to the bottom of the gradient contained predominantly unlysed cells, usually in the dividing state. Interposed between the two bands was a turbid region which contained a mixture of large cell debris, incompletely emptied lysed cells, and intact, single cells. The contents of the bands were drawn-off separately, resuspended in buffer, centrifuged, and suspended in AVO buffer.

Samples of whole and lysed cells were fixed with 1% osmium tetroxide buffered at pH 6.1 with Veronal acetate buffer as described by Kellenberger et al.
Fig. 1–4. Sections of Azotobacter vinelandii growing exponentially in air (N\textsubscript{2}), 0.25\% NH\textsubscript{4}Cl, 0.25\% NaNO\textsubscript{3}, and 0.5\% Casamino Acids, respectively. Visible are the cell wall (CW), the cell membrane (CM), the internal membrane system (IM), the nuclear region (NR), a microtubule (MT), and poly-\(\beta\)-hydroxybutyrate (PHB) granules. The bar in Fig. 1 represents 1 \(\mu\)m. \(\times\) 28,000.
Fixed preparations were dehydrated in a graded series of mixtures of ethyl alcohol and propylene oxide. Fractions were then embedded in Epon 812 by the method of Luft (14). The blocks were cured for 48 hr at 60 C. Thin sections of the cured blocks were cut on a Reichert OmU2 automatic ultramicrotome with a DuPont diamond knife and were mounted on Formvar, carbon-coated grids. Sections were doubly stained, first in 2% uranyl acetate for 2 hr and then in lead citrate according to Reynolds (20) for 5 min. After staining, samples were examined with an RCA electron microscope (EMU-3F) with a double condenser fitted with a 50-μm objective aperture, operated at 50 kv.

RESULTS

Figures 1 through 4 are photographs of thin sections of whole cells of Azotobacter grown with air (N₂), ammonia, nitrate, and amino acids, respectively. Several striking features are apparent. First, the cytoplasm of the N₂-grown cells contains a vast internal membranous network. Cells grown with ammonia or amino acids contain minimal quantities of internal membrane primarily concentrated at the cell periphery. Nitrate-grown cells lack internal membrane completely. Secondly, the cells grown with N₂ are more dense; e.g., the cytoplasmic contents appear to be packed more tightly than cells grown with ammonia or amino acids, although cells grown on nitrate approach the N₂-grown cells in density. Finally, cells grown with atmospheric nitrogen are smaller than cells grown with ammonia. Dimensions of the former average 2.5 by 1.5 μm, whereas those of the latter average 3.1 by 2.0 μm. Cells grown with nitrate are intermediate in size between the extremes of N₂-grown and ammonia-grown cells.

The doubling times for cultures of Azotobacter grown with ammonia, amino acids, nitrate, and N₂ were 1.5, 1.7, 1.8, and 2.0 hr, respectively,
corresponding to growth rates \( k \) of 0.46, 0.40, 0.38, and 0.34.

For a clearer demonstration of the internal membrane system within Azotobacter, cells were lysed osmotically to remove the readily extractable cytoplasmic constituents. Lysed cells were separated from whole cells by the use of a modified sucrose gradient. The internal membrane system of Cells grown in N\(_2\) became obvious (Fig. 5). It consists of invaginating, elongated tubules with alternating bulbous and constricted regions, originating from the peripheral cytoplasmic membrane, which are partially obscured by the cytoplasm in the intact cell (Fig. 1). The internal organization of these membranes closely resembles that described by Holt and Marr (9) in Rhodospirillum rubrum. Figure 9 is a schematic representation of the internal membrane system and the cell wall structure in lysed Azotobacter.

Figure 6 shows a thin section of an osmotically lysed ammonia-grown cell. In such cells, there is a small amount of the membrane network; what is present is concentrated only around the internal periphery and is rarely seen protruding into the interior of the cell. A regular observation in the ammonia-grown cells is a pool of electron-dense, crystalline material which is not readily extruded upon cell lysis. Presumably this material is poly-\( \beta \)-hydroxybutyrate, which appears as the large "lipoidal" bodies (27) in the unlysed cells. The crystalline appearance probably results from fixation or dehydration of the lysed cells, or from both.

Cells grown with nitrate contain little if any peripheral internal membranes (Fig. 7), although their occurrence in amino acid-grown cells varies according to the batch of amino acids used (Fig. 8). Poly-\( \beta \)-hydroxybutyrate does not seem to be synthesized when nitrate or amino acids are used as the nitrogen source. Poly-\( \beta \)-hydroxybutyrate may act as an electron sink in ammonia-grown cells. The polymer is not formed.
Fig. 9. Schematic representation of the cell wall and internal membrane system in *Azotobacter*.

in nitrate-grown cells because the nitrate itself acts as an electron sink.

Figures 5 to 8 also show that a portion of the ribosomes remain within the cell after osmotic lysis. These ribosomes-polyribosomes are probably attached to an ill-defined monolayered reticulum, which transverses through the cytoplasm region, rather than to the internal membranous network (Fig. 9).

**DISCUSSION**

The most significant observation is that the extensive internal membranous network in *Azotobacter* is synthesized only when the cells are grown with air as the sole nitrogen source. There are several possible reasons for the formation of this additional quantity of membranes when *Azotobacter* species are fixing nitrogen. First, since it is generally accepted that the nitrogenase system is particulate, the nitrogenases may be embedded in, or be a constituent of, the membrane. Thus, the internal membrane network is formed concomitantly with nitrogenase induction. Secondly, the respiratory activity of the cell membranes may be required in order for the cell to cope with the already known severe oxygen lability of the nitrogenases.

*Azotobacter* cells grown with ammonia or amino acids are larger than their counterparts grown with atmospheric nitrogen. It is questionable whether this is simply a reflection of an identical cytoplasmic complement packed into a smaller volume because of the vast internal membrane network.

Cells utilizing ammonia grow 33% faster than those using N₂. A faster growing culture would be expected to contain smaller cells rather than larger ones, as found experimentally.

Growth of *Azotobacter* in Burk's medium supplemented with amino acids is enigmatic. It is not at all clear whether these microbes use the full complement of amino acids per se, a select few, e.g., glutamate and aspartate, or, finally, simply deaminate selected amino acids and proceed as if limited by ammonia. According to these experiments *Azotobacter* does not seem to be inhibited by the presence of the amino acids.

Perhaps some batches of protein digest allow deamination and thereby the ultrastructure takes on the appearance of ammonia-grown cells. In other instances, a particular lot of protein digest may not contain the correct amino acids in the correct concentration; therefore, the cells grow under nitrogen-fixing conditions and demonstrate the corresponding ultrastructure. In the former case, ammonia must be limiting, since poly-β-hydroxybutyrate is not synthesized.

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**LITERATURE CITED**


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